HISTOLOGIC DIFFERENTIATION BY MEANS OF ANILIN STAINS IN ASSOCIATION WITH "REGRESSIVE MORDANTING," WITH ESPECIAL REFERENCE TO ELASTIC TISSUE *

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It is well-known that a number of substances have the power of mordanting stains in animal tissues, and that this result may be brought about, in some cases, by applying the two reagents mixed together, while in others, by the mordants following the dye, or vice versa. In fact, it may be said that a great majority of our staining methods depend on some such action, and that we unquestionably owe our most useful histologic differentiations to a combination of stains and their proper mordants. In view of the foregoing facts, it occurred to me some years ago that it might be possible to employ some of the latter substances before staining and by means of appropriate reagents withdraw them from those structures with which they were only feebly combined, and then secure, by appropriate stains, differentiation of those elements with which they were in more stable union.

As I am aware of no work having been done previously along these lines, I have ventured, for obvious reasons, to call the process "regressive mordanting."

It first became necessary to investigate the action of mordants on tissues fixed in different ways. It was found that elastic tissue exhibits a decided tendency to retain two, at least, different mordanting substances, but formalin fixation is necessary to secure the best results.

It was found that permanganate of potassium and phosphomolybdic acid readily lend themselves to the differentiation of elastic tissue, causing it to become basophilic.

Naturally, the substances used to abstract the mordant from the tissues, with which it is more feebly combined, vary with each reagent employed. With permanganate of potassium it was found that reducing agents readily produce satisfactory results, while, with phosphomolybdic acid, alkalies prove eminently satisfactory.

* Received for publication August 29, 1914.

For the differentiation of elastic tissue by this process, the following methods were employed:

Fixation in formalin, followed by dehydration, clearing, embedding in paraffin, and cutting processes, and finally fixation of the sections to the slides by means of Mayer's albumin.

After the paraffin is dissolved out of the sections, they are placed in a 1 percent solution of either potassium permanganate or phosphomolybdic acid; in the former, they should remain only a minute or so, as otherwise the tissues become deeply stained, while, in the latter, they may be allowed to soak from a few minutes to twenty-four hours. The sections are then washed in water and, if the potassium permanganate method is employed, placed for a minute in a 1 percent solution of sodium thiosulphate or oxalic acid in water. When the phosphomolybdic acid has been used, they are immersed for about one minute in a solution of 1/10 normal sodium hydroxid, diluted three or four times with water. Unfortunately the strength of the sodium hydroxid solution is often sufficient to cause the sections to come off when Mayer's albumin has been employed for fixing them on the slides; under such circumstances, the tissues may be protected by immersing the sections for an instant in an extremely weak solution of celloidin, and then transferring them to water. This causes the precipitation of a thin film of this substance, which acts as an effective support and permits their being carried through the alkaline solution without injury. This trouble may be likewise obviated by placing the sections in an incubator for twenty-four hours after fixation to the slides.

The section may now be stained, and it will be found that the elastic tissue is very sharply differentiated by basic anilin dyes; this process is extremely rapid, requiring from a few seconds to a minute or so. After the section is completely stained it is usually only necessary to wash it well in alcohol, which removes the excess of stain and dehydrates at the same time, tho, in other instances, differentiation may be only brought about satisfactorily by using slightly acidulated alcohol. The necessity for differentiation largely depends on how thoroughly the mordant has been abstracted from the other structures; should it only be partially withdrawn all of the tissues stain, tho the elastin much more deeply. In this way an excellent counter staining effect is obtained.

Perhaps the most satisfactory of all of the dyes that may be employed is victoria blue in watery solution, in which the sections remain only for a few seconds, are then washed in water, dehydrated, and mounted in the usual way. Where the mordant has not been entirely removed from the other tissues, a particularly satisfactory stain is obtained by allowing the sections to remain for some minutes in a 1 percent solution of victoria blue dissolved in a 1/1,000 aqueous solution of oxalic acid — little or no differentiation being necessary when this method is used. In sections stained in this way it will be found that the elastic tissue fibrils are colored intensely blue, the epithelial structures not so decidedly blue, while the remaining tissues show shades of the same color, depending upon how thoroughly the mordant has been removed, the length of time which the sections remain in the staining solution, and the degree of final differentiation. Another very satisfactory way of employing this method is to stain with carbol fuchsin, particularly after phosphomolybdic acid. In this case the staining process should be somewhat prolonged, requiring 1.5-2 minutes, followed by washing out in alcohol very slightly acidulated with some acid, great care being taken not to carry the differentiation too far. Unfortunately, the carbol fuchsin

method appears to be somewhat capricious, as oftentimes the finest fibrils are not stained. This is due to over-differentiation. Where the method is properly carried out, the results are very effective. I have found it of advantage, after differentiation is completed, to immerse the section for a few moments in the sodium hydroxid solution and then rapidly dehydrate and clear; this is particularly desirable where acid solutions have been used, as otherwise the colors fade. In addition to the stains mentioned, other anilin dyes give excellent results, for instance, thionin, toluidin blue, methylene blue, azure, neutral red, safranin, magdala red, crystal violet, gentian violet, etc. Carbol toluidin blue gives particularly pleasing effects, as does also thionin; when the latter reagent is employed differentiation with acid solutions produces striking results, the tissues generally retaining more or less of a blue color, and the elastic fibrils showing a bluish purple tinge — this being particularly marked after oxalic or tartaric acid. I would again repeat that, after differentiation is completed, all of these stains appear to be somewhat improved, particularly where acid solutions have been employed for the purpose of differentiation, if the sections are immersed, for a few moments, in a solution of sodium hydroxid.

I have been unable to stain the elastic fibrils with either carmin or haematin. Thus it appears to be possible that the chemical constituents which determine basophilism with anilin dyes are not the same as those which unite with and produce staining effects with the two stains referred to. On the other hand, it is also possible that the mordants used have an extraordinary affinity for basic anilin dyes, as well as elastic tissue, and, clinging to the latter, when these methods are used, attracts and firmly retains the former. In other words, it may be simply a reaction between the mordant and the dye which chances to occur in the elastic tissue, as the former substances are held more tenaciously by elastin than by other tissues. It is further noteworthy that epithelial structures stain readily by this method, the protoplasm retaining the dye in a much more pronounced fashion than the nuclei — thus presenting a reaction which would appear to resemble closely that which occurs in the elastic tissue. This effect is so pronounced that it appears in all of the tissues heretofore examined clearly to differentiate epithelial cells from those of mesoblastic origin, and on further trial may prove to be of value in differentiating carcinoma from sarcoma

As has been before mentioned, counter stains may be employed. Where victoria blue is used, this result may be secured by placing the slide in a watery solution of eosin, picric acid, or acid fuchsin, either before or after coming from the victoria blue bath. Where the tissues are stained with carbol fuchsin, either anilin blue or picric acid gives good results; where the former dye is used the effects are more satisfactory if it precedes the carbol fuchsin; picric acid appears to inter-

fere with the staining in those cases where it is employed before mordanting or just after, and it must, therefore, always follow the basic dye.

The elastic tissue fibrils seem to stain as well, if not better, by Weigert's method after regressive mordanting than without it, while on the other hand they seem to lose almost entirely their capacity to stain by orcein.

These methods clearly show that elastic tissue fibrils may be differentiated into an outer, more intensely staining zone, and an inner zone which, in many instances, fails to take the dye entirely. Where the tissues are properly differentiated, this effect may be observed with the greatest clearness, the outer portion of the elastic tissue fibrils appearing to form what might be called a sheath. This is accentuated by the fact that, in many instances, the fibrils show, for a short distance in either direction from where they are cut in two, a marked increase in the intensity of the peripheral coloration, giving the appearance that the staining solution had penetrated between what might be called the medullary substance and a possible outer sheath. However, I have been unable to definitely determine a physical boundary between the two; in fact the tendency of the stain to show a deeper zone of peripheral intensity of coloration, where differentiation is not carried out so completely, would seem to indicate that the reaction referred to is simply dependent on an increased capacity of the substance of the fibre to retain the stain from within outward.

This method has given extremely gratifying results wherever used, with the exception that both the very fine elastin fibrils and the connective tissue structures, in the outermost layers of the derma, part with the mordant much more readily than do similar structures deeper down in the skin, or even fibrils of elastin of the same size in the walls of the blood-vessels. So readily do these superficicial elastin fibrils give up the mordant that they seem to retain it but little, if any, longer than the deeper connective tissues. The result is that where differentiation is carried on to the extent of completely robbing these tissues of the mordanting substance a similar action has likewise occurred in all of the structures comprising the outermost layers of the true skin. Therefore, when it is particularly wished to study the superficial dermal elastin, the differentiating bath should be materially shortened, in which case, however, the deeper collagenous tissues stain almost as intensely as do the elastic tissue fibrils with which they are closely

associated. Elacin, which is usually so abundantly present in the middle layers of the true skin following pellagraderms, does not appear to be altered in its staining reactions by the mordants employed, tho it parts with basic stains morereadily than does the normal elastin where the pathologic process is advanced. Therefore, it is generally easy to differentiate the normal from the diseased structures. This is an advantage that these methods have over that of Weigert, since elacin reacts with this stain precisely as does normal elastin.

These results warrant further investigation, and it is my hope that some one else may take up the matter and exhaust its possibilities.